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Autoregulatory Frameshifting in Decoding Mammalian Ornithine Decarboxylase Antizyme

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Summary

Rat antizyme gene expression requires programmed, ribosomal frameshifting. A novel autoregulatory mechanism enables modulation of frameshifting according to the cellular concentration of polyamines. Antizyme binds to, and destabilizes, ornithine decarboxylase, a key enzyme in polyamine synthesis. Rapid degradation ensues, thus completing a regulatory circuit. In vitro experiments with a fusion construct using reticulocyte lysates demonstrate polyamine-dependent expression with a frameshift efficiency of 19% at the optimal concentration of spermidine. The frameshift is +1 and occurs at the codon just preceding the terminator of the initiating frame. Both the termination codon of the initiating frame and a pseudoknot downstream in the mRNA have a stimulatory effect. The shift site sequence, UCC-UGA-U, is not similar to other known frameshift sites. The mechanism does not seem to involve re-pairing of peptidyl-tRNA in the new frame but rather reading or occlusion of a fourth base.

Introduction

In decoding certain mRNAs, a high proportion of ribosomes shift from the initiating reading frame to one of the other two frames in response to signals in the mRNA. This programmed ribosomal frameshifting constitutes an important part of recoding or reprogrammed genetic decoding (Gesteland et al., 1992). Programmed frameshifting is well known in the decoding of compact genomes such as retrotransposons and bacterial, yeast, plant, and animal viruses (especially retroviruses), but only a few cases are known in cellular genes. The classic case of the latter is the autoregulatory frameshifting involved in decoding the Escherichia coli release factor 2 (*RF2*) gene (Craigen and Caskey, 1986; Weiss et al., 1987; Curran and Yarus, 1988). Until now, programmed frameshifting was unknown in decoding animal genes. We describe the autoregulatory involvement of frameshifting in decoding mammalian antizyme and show that not only is the frameshifting different from that used by mammalian viruses, it is distinct from all other known types of frameshifting.

Antizyme is a polyamine-inducible protein that binds to, and inhibits, ornithine decarboxylase (ODC) (reviewed by Hayashi and Canellakis, 1989). ODC is a key enzyme for polyamine biosynthesis in animal cells (reviewed by Pegg, 1986). It converts ornithine to a diamine, putrescine, which in turn leads to spermidine and spermine. ODC has the fastest turnover rate of any mammalian enzyme, with a half-life of 10–60 min (Russell and Snyder, 1969). Elevation of cellular polyamine levels accelerates degradation of ODC (Hayashi, 1989), and early results suggested that antizyme is essential in this process (Murakami et al., 1985; Murakami and Hayashi, 1985). This was confirmed by using antizyme expressed from a cDNA clone (Murakami et al., 1992a, 1992b), and the degradation was shown to be catalyzed by 26S proteasome without ubiquitination (Murakami et al., 1992c; Tokunaga et al., 1994). The C-terminal half of antizyme binds to ODC, inducing a conformational change (Li and Coffino, 1993), allowing an additional internal sequence in antizyme to promote destabilization of ODC (Li and Coffino, 1994; Ichiba et al., 1994). Consequently, ODC is not only short-lived like other proteins that control important cellular processes (Goldberg and St. John, 1976), but its turnover is also regulated. Though mechanistically distinct, the induced destabilization by antizyme is analogous to that of human papillomavirus oncoprotein E6 action on the tumor suppressor p53 (Scheffner et al., 1990). Antizyme has a second function: it also represses polyamine uptake, thereby enabling sharper shutdown of polyamine accumulation in cells (Mitchell et al., 1994; Suzuki et al., 1994).

Several features of antizyme induction by polyamines point to regulation of antizyme translation. Antizyme synthesis is inhibited by cycloheximide but not actinomycin D (Fong et al., 1976; Matsufuji et al., 1990a). While only a minute amount of antizyme is present in mammalian tissues (around 1 ppm of soluble protein), its mRNA level is relatively high and not further elevated by polyamines (Matsufuji et al., 1990b). Polyamines interact with nucleic acids and are important for protein synthesis, particularly for accurate decoding (Atkins et al., 1975; reviewed by Tabor and Tabor, 1984). However, the polyamine action on antizyme translation is specific for frameshifting. The mechanism involved is addressed here.

A partial cDNA clone for antizyme, Z1, has been isolated (Matsufuji et al., 1990b). Although Z1 lacks an in-phase AUG initiator along with its 5′ untranslated region and some 5′ part of the coding region, its open reading frame (ORF) potentially encodes active antizyme. This was shown by production of active and immunoreactive protein from a derivative of the cDNA supplemented with an appropriately placed *lac* promoter and a translational initiator in E. coli (Matsufuji et al., 1990b). Active antizyme was also produced in transfected mammalian cells from the cDNA driven by mouse mammary tumor virus long terminal repeat promoter (Murakami et al., 1992a). In this cell line, antizyme synthesis is dependent on dexamethasone but is not further stimulated by polyamines, implying that the

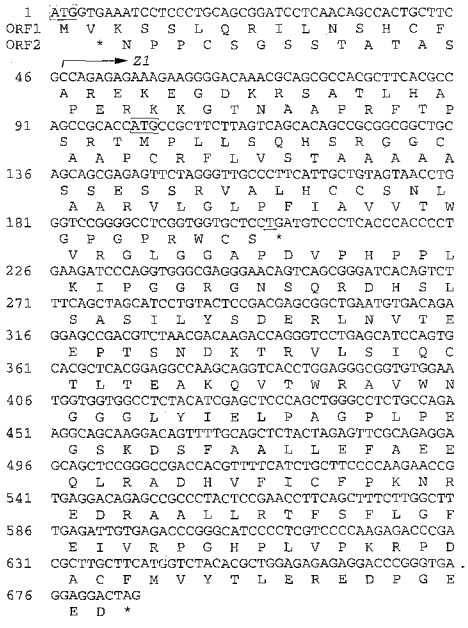

Figure 1. Nucleotide Sequence of Antizyme-Coding Region and Potential Amino Acid Sequences

Asterisks denote the termination codons. Nucleotides are numbered starting from the first ATG. All of the antizyme constructs described follow this numbering system. Two initiator codons are boxed. The underlined nucleotide was deleted in the in-frame control, ΔT205. The 5′ endpoint of the partial-length cDNA (Z1) is shown.

proximal gene segment missing from Z1 is important for the translational regulation by polyamines. To determine the entire mRNA sequence, a genomic clone was isolated (Miyazaki et al., 1992) in parallel with full-length cDNA clones (see Results). As reported here, an analysis of these clones revealed that antizyme is decoded from two ORFs by ribosomal frameshifting that is dependent on polyamine levels. These conclusions were reported previously (Gesteland et al., 1992). Rom and Kahana (1994) reported polyamine-dependent frameshifting of antizyme expression using one of these same clones.

Results

Expression of Antizyme mRNA Requires Frameshifting

Two full-length cDNA clones were isolated from a rat liver library and sequenced. They are identical except that they have different 5′ endpoints, and they contain the exact sequence present in the partial cDNA clone Z1 (see Introduction). Their sequence is also identical with that of the exons in the genomic clone that was determined by S1 analysis with the partial cDNA clone (Miyazaki et al., 1992). This result is consistent with the lack of editing of the transcripts.

Two major ORFs are evident (Figure 1). ORF1, beginning at the first AUG, has a length of 204 nt terminating with UGA and could encode a polypeptide of 7441 Da. It is not sufficient to encode antizyme whose relative molecular mass (M_r) is 18–19 kDa as determined by SDS–PAGE on purified preparations (Kitani and Fujisawa, 1984; Matsufuji et al., 1990a). ORF2 overlaps most of ORF1 by 198 nt and consists of 675 nt (adequate to encode a protein of 24,321 Da) but does not have an obvious initiation codon; its sole AUG is 14 codons from the 3′ end. The original cDNA clone, Z1, contained most of ORF2, which, when experimentally expressed, produced active protein (see Introduction).

There are several possible explanations for the expression of antizyme from this mRNA, including unusual ribosome initiation, RNA editing, and posttranscriptional modification such as transpeptidation. However, there is an increasing number of examples of programmed ribosomal frameshifting to express one protein from overlapping ORFs. The experiments presented here address the frameshift model and rule out the other possibilities, as discussed below.

To investigate frameshifting, the complete two ORFs were inserted downstream of bacteriophage T7 RNA promoter so that in vitro synthesized RNA could be made and then translated in rabbit reticulocyte lysates. As a control, an in-frame construct, ΔT205, was prepared by deleting the T at nucleotide 205 (underlined in Figure 1), which removes the stop codon at the end of ORF1, creating a continuous ORF with the whole ORF1 and the nonoverlapping part of ORF2. The proteins synthesized with or without the addition of spermidine were compared because of the unusual involvement of polyamines in antizyme expression in vivo.

[^{35}S]Methionine-labeled products were analyzed by SDS–PAGE (Figure 2A). Translation of the control mRNA with ORF1 and ORF2 in the same frame resulted in two major products (of M_r 29 kDa and 23 kDa), and their synthesis was not stimulated by spermidine. The same two products are seen from the wild-type mRNA (ORF1 and ORF2 out of frame), but spermidine greatly enhanced the appearance of both proteins. The gel patterns are the same with or without precipitation with antizyme antibody (data not shown).

The two products from both wild-type and in-frame mRNAs result from initiation at the first and second (nucleotides 100–102) AUG codons in ORF1 (boxed in Figure 1). This is shown by the following two experiments. With mutational analysis (Figure 2B), elimination of the first AUG (AUG1) abolished the 29 kDa band (this change also enhanced a band of ~26 kDa, probably through increasing a non-AUG initiation at an unknown position). Elimination of the second AUG (AUG2) abolished the 23 kDa band. N-terminal sequence analysis of the two proteins by Edman degradation of [^{3}H]leucine- or [^{3}H]valine-labeled material gave sequences Val-X-X-X-Leu-X-X-X-Leu-X-X for the 29 kDa band and X-Leu-Leu-X-X-X-X-X-X-X-X (no valine) for the 23 kDa band. These are consistent with the predicted sequences with removal of the N-terminal methionine. Initiation of translation of the wild-type mRNA at these two AUG codons, terminating at the in-frame UGA, should give polypeptides of 67 and 34 amino acids (7311 and 3570 Da, respectively); at least the former should be detected by the gel assay but was barely seen (data not

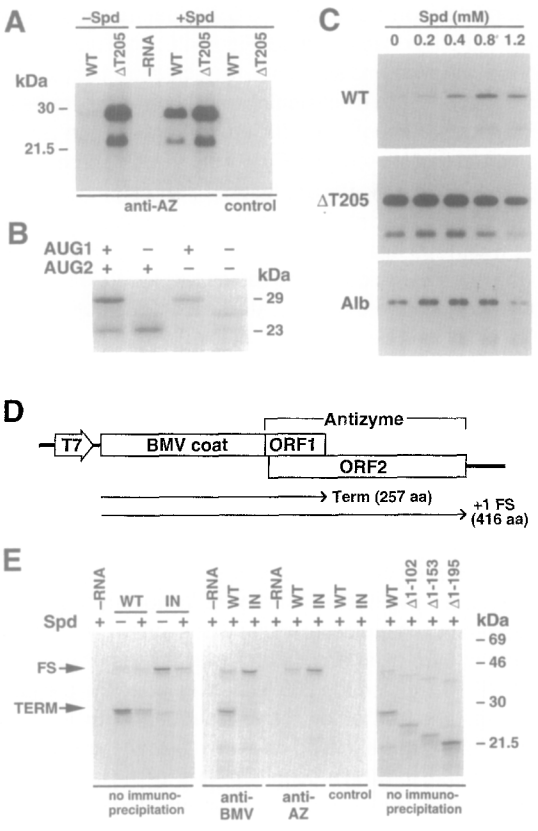

Figure 2. Translation of Antizyme mRNAs in Rabbit Reticulocyte Lysate

(A) The wild-type (WT) and ΔT205 mRNAs were translated with (+Spd) or without (–Spd) addition of 0.8 mM spermidine. The products were immunoprecipitated with the anti-antizyme or preimmune IgG (control), separated on 15% SDS–PAGE, and processed for fluorography. The positions of M_r markers are indicated on the left.

(B) Mutational analysis of AUG codons. Each or both of the two AUG codons of ORF1 located at nucleotides 1–3 and 100–102 (boxed in Figure 1) was changed to AUC and translated as above in the presence of 0.8 mM spermidine.

(C) Effect of spermidine on translation in the rabbit reticulocyte lysates. The wild-type and ΔT205 mRNAs were translated in the presence of indicated concentrations of exogenous spermidine. Translation of albumin (Alb) RNA from rat liver poly(A)$^+$ RNA was also tested with the same conditions.

(D) Diagram of BMV coat–antizyme fusion construct with the wild-type antizyme (wild-type fusion). Essential components and expected lengths of the products of termination (TERM) and +1 frameshifting (FS) are indicated. T7 denotes the T7 promoter.

(E) Autoradiograms of translation products from fusion constructs. Left, the wild-type (WT) or in-frame (IN) fusion mRNA was translated in a reticulocyte lysate supplemented with (+) or without (–) 0.6 mM spermidine (Spd) and fractionated without immunoprecipitation. Center, translation products synthesized in the presence of 0.6 mM exogenous spermidine were immunoprecipitated with antibody against BMV or antizyme (AZ), or preimmune IgG (control). Right, translation of 5′ deletion mutants of antizyme. Constructs were translated in the presence of 0.6 mM exogenous spermidine and separated without immunoprecipitation. All the protein products were separated on 12.5% SDS–PAGE. The positions of the frameshift (FS) and termination (TERM) products are indicated.

shown), suggesting that it may be rapidly degraded in reticulocyte lysates. (However, this region of the gel is obscured by the great excess of globin in reticulocyte lysates. A 7 kDa protein can be discerned if the wild-type RNA is translated in extracts from wheat germ [data not shown].)

Translation products of these mRNAs do contain the ORF2 sequence: they react with an antibody specific to the bacterial ORF2 product (Figure 2A), and they exhibit antizyme activity by binding to, and inhibiting, ODC (data not shown), which is known to be encoded wholly within the nonoverlapped part of ORF2 (nucleotides 206–682) (Matsufuji et al., 1990b; Murakami et al., 1992a). These findings, taken together with the determination of ORF1 N-terminal sequences, indicate that the reading frame for antizyme translation changes from ORF1 to ORF2 at some position within the overlap of the two ORFs. ORF2 does not appear to be expressed independently of initiation at ORF1, arguing against subsequent transpeptidation of ORF1 and ORF2 products.

Spermidine affected synthesis of antizyme products from the wild-type RNA. This preparation of reticulocyte lysate endogenously contained 0.36 mM spermidine and 0.03 mM spermine as final concentrations for translation. At the optimal concentration of 0.8 mM of exogenous spermidine, there is a 10-fold stimulation in antizyme synthesis over the level seen with no added spermidine, bringing the efficiency up to about 30% of the in-frame control (Figure 2C; comparison with the in-frame control is necessary, since the small non–shift termination product cannot be detected). In contrast, translation of the in-frame mRNA was efficient even at low spermidine concentrations and was somewhat suppressed by increasing concentrations of spermidine. This inhibition is nonspecific, as translation of albumin mRNA shows the same effect. Spermine and putrescine also stimulated synthesis of antizyme from wild-type mRNA, with optimal concentrations of 0.12 mM and 4 mM, respectively, whereas magnesium (1–4 mM) did not show the effect (data not shown). These data show that the polyamine stimulatory effect is specific to translation of the wild-type mRNA, which requires an unusual translocation event. Presumably the ribosome initiation rate is the same on both mRNAs, and it is the frameshift event that responds to polyamine addition.

To reveal the product of the wild-type mRNA that terminates at the UGA in ORF1 and to get a measure of the efficiency of transition from ORF1 to ORF2, a fusion construct was made with the coat protein gene of brome mosaic virus (BMV) 5′ to the antizyme gene (Figure 2D). Ribosomes initiate at the BMV AUG and terminate at the ORF1 UGA to make a protein of 257 amino acid residues and a mass of 27,832 Da. If they switch to the +1 frame ORF2, they will make a full-length product of 416 amino acids with an approximate mass of 46 kDa (the exact mass will depend on where the shift in frame occurs, since this will change the amino acid composition). As shown in Figure 2E (WT, no immunoprecipitation), two products of the anticipated sizes were synthesized from mRNA of this fusion construct. The larger protein was the same size as the main product of the in-frame control (Figure 2E, IN). Both products in the wild-type fusion construct were immuno-

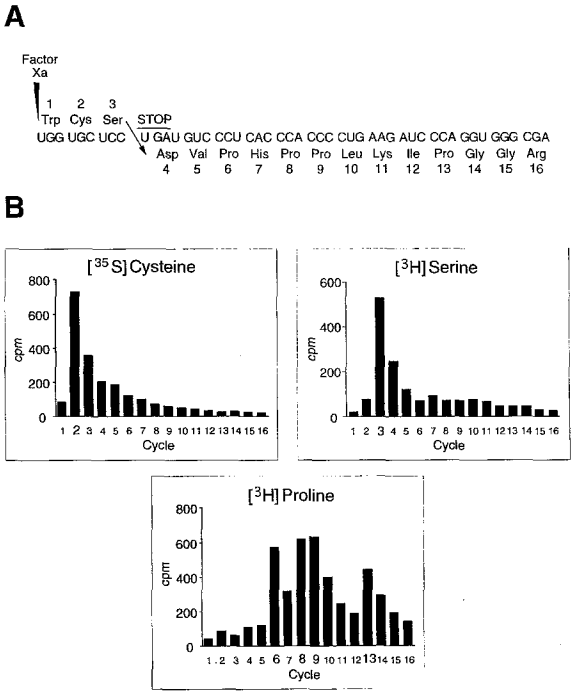

Figure 3. Determination of Amino Acid Sequence of the Transframe Peptide Isolated from an In Vitro Translation Product

(A) Relevant nucleic acid sequence and predicted amino acid sequence. The sequence following the 5′ factor Xa cleavage site is shown with residue numbers.

(B) Amounts of labeled amino acids detected in each cycle of Edman degradation. [^{35}S]Cysteine and [^{3}H]proline were used in one experiment and [^{3}H]serine in the other (peptide purification to be published elsewhere).

precipitated with anti-BMV antiserum, and only the larger product reacted with the antibody specific to ORF2 of antizyme (Figure 2E, center). These results ensure that the full-length product of the wild-type fusion is translated from two different reading frames and strongly suggest frameshifting into the +1 frame. The efficiency of the shift can be determined from the ratio of radioactivity of the terminated and full-length products after calibrating the numbers of amino acid residues labeled in the two products. Spermidine has a nonspecific inhibitory effect on translation, so it is necessary to normalize the results to the total translation observed. The absolute amount of the frameshift product did not increase significantly and even decreased with higher concentrations of spermidine. After normalization, the shift efficiencies were 5.4% without polyamine addition and 19.0% with addition of 0.6 mM spermidine. (There is variability in the quantitative polyamine stimulation, probably reflecting differences in reticulocyte lysates and the nature of the 5′ sequences in the mRNA. More experiments are needed to clarify these issues.)

5′ Deletion Analysis

Signals for programmed frameshifting generally consist of the actual shift site plus nearby *cis*-acting sequences in the mRNA that stimulate the frameshift (Atkins et al., 1990). To narrow the regions containing these signals, several deletion mutants of the BMV coat–antizyme fusion were tested for frameshifting (Figure 2E, right panel). Deletions of the antizyme sequence to nucleotide 102 (Δ1–102) or 153 (Δ1–153) did not significantly change the frameshift efficiency. Although the efficiency dropped, the Δ1–195 construct that lacked all of ORF1 but the last three codons still exhibited a significant level of frameshifting (38% of the wild-type fusion). This result puts the likely frameshift site within the last three codons of ORF1, just at the end of the overlap region. It also raises the possible existence of a stimulatory element located within nucleotides 154–195, which will be discussed below.

Amino Acid Sequence of the Transframe Peptide

To pinpoint the change in reading frame, the amino acid sequence was required. However, sequencing of antizyme from cells is difficult, because the protein is present in only very small amounts. Simple N-terminal sequencing of radiolabeled, in vitro synthesized protein is difficult, since the predicted shift site is so far into the molecule. Instead, an engineered form of radiolabeled protein synthesized in a reticulocyte lysate was sequenced. RNA was made in vitro from a construct with the last four codons of ORF1 (to nucleotide 268, which includes the presumed shift site and all the downstream sequence necessary for the efficient frameshifting as shown below) flanked by two cassettes specifying recognition sites for the factor Xa protease, which cleaves on the C-terminal side of Ile–Glu–Gly–Arg. The 5′ factor Xa site was positioned such that three amino acids remain from ORF1 after cleavage (Figure 3A). The 3′ cleavage site was positioned to reduce the size of peptide to be sequenced, thereby minimizing the background during Edman degradation. The mRNA was translated in a reticulocyte lysate in the presence of both [^{35}S]cysteine and [^{3}H]proline and, in a separate experiment, [^{3}H]serine. The frameshift products together with the termination product were purified by glutathione–Sepharose column chromatography and digested with factor Xa. The transframe peptides were then isolated with reversed-phase high performance liquid chromatography and subjected to automated Edman degradation (Figure 3B). Cysteine was found in cycle 2, serine at cycle 3, and proline in cycles 6, 8, 9, and 13, to give the sequence X-Cys-Ser-X-X-Pro-X-Pro-Pro-X-X-X-Pro. Factor Xa cleavage at arginine is predicted to leave cysteine as the second amino acid and serine as the third in the zero frame as found. The prolines at 6, 8, 9, and 13 are all predicted by the +1 reading frame, and therefore the frameshifting must be +1 but not −2 or +4, and so forth. Readthrough of the UGA terminator is ruled out, since cysteine (specified by UGU at the next position to the terminator in the zero frame) is not found in cycle 5. The reading frame must have changed either at the serine codon before the terminator or at the terminator: the later case could be achieved only if the terminator is decoded as a sense codon followed by immediate frameshifting, but this seems unlikely, as it requires two nonstandard events. We conclude that the last codon of ORF1, UCC, is the site of the +1 change in reading frame. While the results all point to ribosomal frameshifting, editing out of one base in the mRNA at this

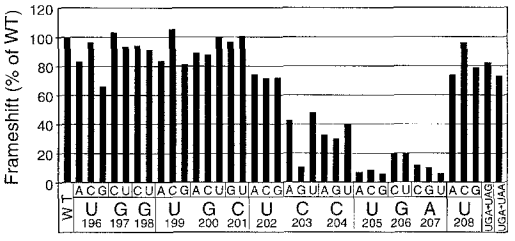

Figure 4. Mutational Analysis of the Frameshift Context

The wild-type (WT) sequence, separated as codons, is shown in the bottom boxes. Each nucleotide or the termination codon was mutated to ones indicated above. Translation was carried out in the reticulocyte lysate supplemented with 0.6 mM spermidine. Frameshift efficiencies relative to the wild type were calculated from data obtained by phosphorimaging, normalizing to the sum of the termination and frameshift products, taking into account the number of methionine residues. The values are averages from at least two clones. The frameshift efficiency of the wild-type construct was 17.7% ± 1.4%.

site cannot a priori be discounted. Direct examination of the sequence of mRNA after protein synthesis showed no editing (less than one tenth of the required efficiency).

Mutational Analysis of the Frameshift Context

Various mutations in the frameshift region of the BMV coat–antizyme fusion construct were compared for frameshift efficiency in a reticulocyte lysate. Deletions of each one of the last three codons of ORF1, namely, Δ196–198, Δ199–201, and Δ202–204, gave frameshift efficiencies of 46%, 37%, and 14% of the wild-type fusion, respectively. Substitutions for each nucleotide in this region were also tested (Figure 4). Any mutations of nucleotide 201 or 5′ to it, i.e., upstream of the last codon of ORF1, did not substantially affect the frameshift efficiency, except changing U196 to a G, which decreased frameshifting and incidentally created a run of five Gs. Surprisingly, the first position of the last codon, U202, could also be changed with no more than 30% decrease in frameshifting efficiency. Changing the first codon base alters the tRNA specified, indicating that more than one tRNA can cause this frameshifting. Substitutions at the second and third positions of the last codon resulted in a greater decrease, and the mutation of C203 to a G particularly diminished the frameshift activity, consistent with involvement of these bases in tRNA interaction in the frameshift event.

The results from sequence changes 5′ to the shift site suggested that there may be an unidentified stimulatory element. The C201 to G mutation did not show any effect and creates a context with UGG immediately 5′ to the UCC shift site. However, the mutant Δ199–201, described above, also brings UGG adjacent to the shift site UCC and has 37% activity. Furthermore, the U196 to C mutation creates the sequence CGG UGC UCC and has 82% activity, whereas the Δ196–198 mutant creates the sequence CGG UGC UCC and has 46% activity. These results are difficult to explain, in connection with the decrease of the frameshift activities in the Δ1–195 construct described previously, but may suggest an additional sequence element for efficient frameshifting 5′ to the nucleotide 195, whose distance to the shift site is crucial.

Downstream Stimulators

When a shift site and a termination codon are immediately adjacent, the termination codon often acts as a stimulator in programmed frameshifting (Weiss et al., 1987; Curran and Yarus, 1988; Brault and Miller, 1992) and in low level frameshifting seen with test constructs (Weiss et al., 1990). As shown in Figure 4, any substitution of the UGA termination codon with sense codons severely diminished frameshifting. On the other hand, changing it to the other termination codons, UAG and UAA, only slightly decreased the frameshift, to 82% and 73%, respectively, of the wild-type fusion. Replacement of the nucleotide immediately 3′ to the stop codon did not alter the shifting significantly. However, there may be a mild preference for U or C rather than A or G at this position. These results show that the termination codon of ORF1 is one of the important stimulators. In all the mutants of the termination codon tested, the frameshifting was increased by spermidine addition (data not shown).

Stimulators for −1 frameshifting, particularly in coronaviruses and retroviruses, are often pseudoknots located downstream of the shift site (Brierley et al., 1989; ten Dam et al., 1990; Chamorro et al., 1992). The tertiary structure of a pseudoknot has a stem–loop with nucleotides of the loop region paired to nucleotides downstream of the stem to form a second stem (Pleij et al., 1985). Antizyme mRNA sequence reveals a potential pseudoknot just 3′ to the termination codon of ORF1 (Figure 5A), even though so far no examples of +1 frameshifting are known to be accompanied by downstream RNA structure. The downstream stimulator(s) for frameshifting was delimited by deletion of the sequence 3′ of the pseudoknot with retention of full frameshift activity (Figure 5B, Δ269–682). If the deletion was limited to the potential pseudoknot region (Δ209–268), frameshifting decreased by 60%, suggesting the presence of a stimulator in this sequence. These results combined with the 5′ deletions circumscribed the essential stimulatory signals as being located within the region of nucleotides 205–268. Deletions of various parts of this region also resulted in decreased frameshift activities (Figure 5B), but the roles of potential stems were not clearly delineated, perhaps because of the complexity of other possible conformations.

The stems of the proposed pseudoknot were tested directly by specific mutations. The 5′ side stem (S1) is divided by a pair of bulged-out nucleotides into two 6 nt stems (Figure 5A). Each half of these stems was substituted individually by its complementary sequence to block potential base pairing. For the 3′ side stem (S2), only two nucleotides in the stem were replaced, since changing six nucleotides creates an in-frame terminator. Each of the substitutions resulted in decreased frameshifting by approximately half. When double complementary changes were made so that base pairing was restored, frameshifting was restored. Conversion of the bulging bases in S1 to a GC pair did not alter frameshift efficiency (C-S1). These results provide evidence that the predicted pseudoknot

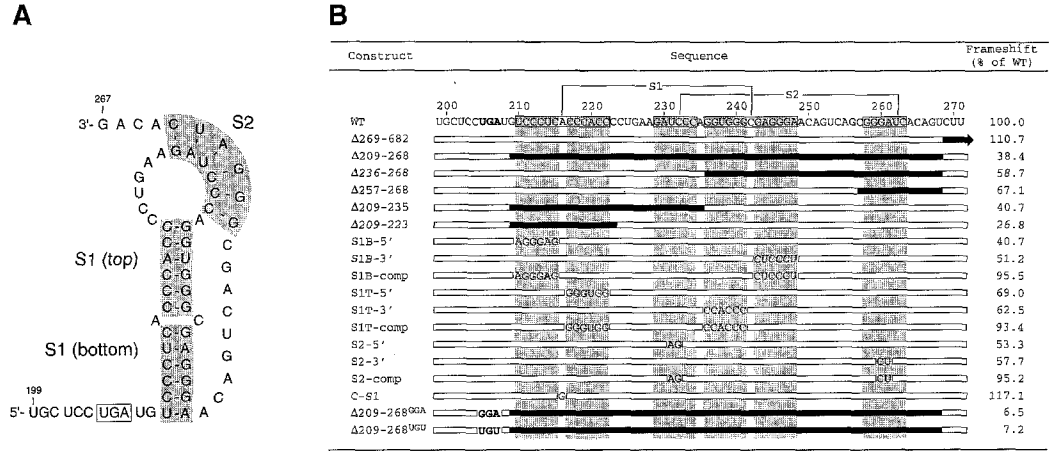

Figure 5. Stimulatory Effect of Downstream Sequence

(A) A potential pseudoknot structure. The termination codon of ORF1 is boxed.

(B) Mutational analysis of the pseudoknot region. Relevant nucleic acid sequence of the wild-type construct (WT) is shown with the stem regions boxed. Open bars of the mutation constructs denote regions identical to the wild-type sequences, and closed bars denote deleted regions. Nucleotide substitutions are indicated. Frameshift efficiencies were measured as described in the legend to Figure 4.

actually forms to enhance frameshifting. Deletion of the pseudoknot region from a construct containing a sense codon in place of the UGA codon does not further decrease the low level of observed frameshifting when compared in the presence of exogenous spermidine (Figure 5B, Δ209–268^{GGA} and Δ209–268^{UGU}). In the absence of added spermidine, frameshifting was not detectable with these mutants (data not shown). Spermidine stimulated frameshifting (approximately 3-fold) whether or not the pseudoknot was present, whether or not the stop codon was present, and with or without deletions 5′ to the stop codon (data not shown).

A Sequence with the Shift Site and the Pseudoknot Directs Frameshifting and Stop Codon Readthrough in a Foreign Context

The results presented above indicate that the essential frameshift signals consist of the last few codons and the stop codon of ORF1 and the downstream pseudoknot. Fusion clones were devised to see whether this region is sufficient for specific +1 frameshifting in a foreign context. These constructs, and rearrangements of them, also test the ability of this region to promote specific −1 frameshifting and stop codon readthrough. A fragment spanning the last five codons of ORF1 and the pseudoknot (nucleotides 190–268) was cloned into a vector, pGB01 (see Experimental Procedures), between an upstream reporter, glutathione S-transferase (GST), and a downstream reporter, rabbit β-globin (Figure 6A). In this construct, pGB-103, the β-globin gene was fused to this fragment of antizyme mRNA so that shifting into the +1 frame was required for its expression. This fusion gene was transcribed with T7 RNA polymerase and translated in a reticulocyte lysate (Figure 6B). The 45 kDa protein that migrated with the product of an in-frame construct results from +1 frameshifting. The 27 kDa termination product corresponds to GST plus the fragment of ORF1. The 31 kDa product is of the expected size for readthrough of the ORF1 UGA terminator. However, −1 frameshifting would give an intermediate-sized protein of 28 kDa. The clone was rearranged in order to focus on −1 frameshifting and readthrough. The β-globin gene was placed in either the zero (pGB-R01) or −1 (pGB-T01) frame with respect to the GST gene (Figure 6B). pGB-R01 encoded two products, a 33 kDa protein predicted from +1 frameshifting and a 45 kDa product from readthrough. pGB-T01 encoded a 31 kDa +1 product and a 33 kDa readthrough product. Neither revealed the predicted −1 frameshift product. The addition of spermidine increased the relative amounts of the frameshift and readthrough products, but with different optimal spermidine concentrations (Figure 6B). At 0.8 mM of added spermidine, +1 frameshifting was 9.9%, compared with 1.0% without added spermidine. For readthrough, maximal efficiency was 8.3% with 0.6 mM of added spermidine compared with 2.6% without. The 79 nt fragment of antizyme is sufficient to promote polyamine-dependent, specific +1 frameshifting when transplanted to a new context, but not −1 frameshifting. The fragment does, however, promote readthrough of its UGA stop codon, but this is unlikely to be of biological importance. (In the BMV coat–antizyme fusion, where the context is more similar to the natural gene, a product with the expected 30 kDa size of that derived from UGA readthrough was not detected.)

Discussion

Regulation of Antizyme Expression

The finding that ribosomal frameshifting is used in expression of a mammalian gene is a major extension of our knowledge of the utilization of recoding. The mechanism for regulation seems to be general: recent results indicate that the antizyme mRNAs from human and Xenopus laevis also require frameshifting for their expression (unpub-

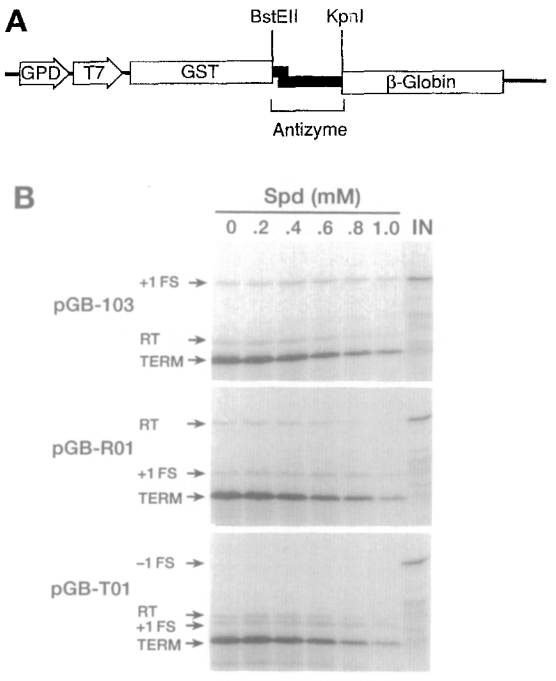

Figure 6. Expression of Constructs with Antizyme Frameshift Cassette in a GST–β-Globin Fusion

(A) Construct used in this experiment. The antizyme sequence, nucleotides 190–268 (solid bar), was inserted into the pGB01 vector. The β-globin frame is +1 (pGB-103), 0 (pGB-R01), or −1 (pGB-T01) with respect to the GST frame. GPD and T7 denote the GPD and T7 promoters.

(B) In vitro translation products. T7-generated RNAs were translated in the presence of indicated amounts of exogenous spermidine, and the products were separated on SDS–PAGE (11%). The positions of +1 and −1 frameshift (FS), readthrough (RT), and termination (TERM) products of each construct are indicated on the left. IN denotes the in-frame constructs, from which the full-length fusion proteins were produced without frameshifting or readthrough.

lished data). The role of this recoding is to provide an elegant autoregulatory circuit. Less is known about an additional regulatory system(s) where antizyme somehow represses a polyamine transporter (Mitchell et al., 1994; Suzuki et al., 1994).

The frameshifting stimulated by polyamines results in an increase in the amount of antizyme, which lowers intracellular polyamine levels by promoting turnover of a key enzyme in polyamine synthesis, ODC. Functional antizyme is encoded in ORF2 alone, and the role of ORF1 seems not to be for its protein product per se, but rather to provide for recoding regulation by polyamines. Cells stably transfected with ORF2 supplied with an in-frame initiator codon showed antizyme activity that was not stimulated by exogenous polyamines (Murakami et al., 1992a). However, the relatively large size of ORF1 (depending on which AUG is used for initiation, 68 or 35 codons) makes it likely that ORF1 is doing more than just providing a ribosome start site in a different frame. In the only other case of autoregulatory frameshifting, E. coli *RF2*, the ORF1 is only 25 codons.

Frameshift Mechanism

Frameshifting for the expression of antizyme in the reticulocyte lysate is +1, occurring at the UCC serine codon immediately before the ORF1 UGA terminator. It is also highly specific. Frameshifting into the other frame (−1) is not detectable.

The known cases of +1 ribosomal frameshifting fall into two general classes: in the first class, the mRNA permits peptidyl–tRNA to slip ahead by 1 base at the shift site and re-pair in the new frame; in the second class, the mRNA allows a translocation step of 4 bases at the shift site. In the antizyme case, re-pairing would require serine tRNA first to pair with UCC, then to move ahead one to CCU, where it could pair with only the last two bases. Imperfect pairs at a re-pairing position are seen in several frameshifting cases (reviewed by Atkins and Gesteland, 1995). However, with antizyme, in vitro mutational analysis shows that re-pairing is not obligatory and with some of the mutants cannot occur. When the UCC is changed to UCG, a significant level of frameshifting (30%) was still observed (Figure 4). This UCG–serine codon is decoded by other serine isoacceptors with anticodons of 3′-AGU-5′ (with or without a modification of the U) or 3′-AGC-5′. Neither of these tRNAs can pair in any of their anticodon positions to the +1 frame, CGU, making re-pairing unlikely. This is further supported by results of changing UCC to ACG, where again no base pairing is possible, yet the reduction in frameshifting efficiency is only 50%. Several possible mechanisms that do not involve re-pairing are compatible with the present data and cannot yet be distinguished (cf. Farabaugh et al., 1993).

Stimulatory Signals

Two stimulatory elements in antizyme mRNA have been identified for mammalian ribosome frameshifting. A stop codon at the end of ORF1 is crucial, but its identity (UAA, UGA, or UAG) is less important. The preliminary evidence here supports the participation of the downstream pseudoknot shown in Figure 5A. While downstream structures have been shown to be stimulatory for −1 frameshifting and stop codon readthrough, this is the first example of involvement in +1 frameshifting, though the 2.5-fold effect is considerably less than is seen in the −1 cases. The spacing between the shift site and the pseudoknot is 5 nt. However, since the UGA codon occupies the A site when the shifting occurs, the relative spacing between the shift site and the pseudoknot is 2 nt. It is much less than the 6–9 nt usually found in −1 frameshifting and readthrough (except prokaryotic selenocysteine [Heider et al., 1992]). However, the existence of the UA pair at the base of S1 may not be concluded from the present data. In addition, two alternative pseudoknots with different S1 can be drawn that would have spacing of 12 nt. One does not have the bottom half of S1. The other has an S1 of seven CG pairs composed of nucleotides 217–224 and 239–247, allowing three bulged-out nucleotides. However, the available data support the model shown (Figure 5A), with short spacing. The results of the compensatory experiments suggest that the primary sequence of the stems of the

pseudoknot region may not be important, similar to the other cases of frameshifting.

Mechanism of Polyamine Stimulation of Frameshifting

It is not surprising that small, polycationic polyamines interact with nucleic acids and have general effects on translation (Atkins et al., 1975; Tabor and Tabor, 1984). In antizyme decoding, polyamines stimulate frameshifting (in vivo and in vitro, at physiological concentrations) to afford autoregulatory control. Whether the polyamine effect is specific for antizyme mRNA or perhaps for all +1 frameshifting is not known. It may be specific for +1 frameshifting, since at least with mouse mammary tumor virus *gag–pro* −1 frameshift, no stimulation is seen (unpublished data). In yeast, mutational alteration of polyamine metabolism influences frameshifting, but effects of specific polyamines remain to be resolved (Balasundaram et al., 1994).

Irrespective of whether the polyamine effect is specific for antizyme mRNA, somehow sequences in antizyme mRNA set up a frameshift event that is responsive to polyamine concentrations. Can a polyamine-specific effector sequence be delimited in the mRNA? One candidate might be the downstream pseudoknot. But constructs without the downstream region are still stimulated by spermidine. Spermidine did not have its effect through the termination codon or upstream sequences, either. This points toward polyamines having their effect through distortion of the decoding site, including perhaps the tRNA, to allow frameshifting. Whatever the detailed mechanism, it does seem remarkable that the sequences in an mRNA can set up a ribosome to respond to the concentration of small, ubiquitous molecules and alter its mode of reading the genetic code.

Experimental Procedures

cDNA Cloning and Sequencing

Oligo(dT)-primed cDNA was synthesized from poly(A)$^+$ RNA from the livers of Sprague–Dawley rats by use of a kit (Boehringer) and inserted into the λZAPII vector (Stratagene) through EcoRI adaptors (Promega) to construct a library. Nine positive clones were selected out of 2 × 10^5 recombinants by plaque hybridization with a probe of the partial length antizyme cDNA Z1 (Matsufuji et al., 1990b). These clones were converted to pBluescript plasmids by in vivo excision. Two of the longest clones, ZZ5 and ZZ4, were sequenced entirely on both strands with synthetic primers or on subclones of restriction fragments. Sequencing was carried out with the dideoxynucleotide chain termination method on double-stranded plasmids.

Plasmid Constructions

The plasmid for in vitro expression of antizyme, termed NE, has three parts: first, a 0.97 kb BamHI–EcoRI fragment of ZZ5 cDNA (containing the nucleotide 24 in Figure 1 and all the following sequence through the 3′ EcoRI linker of the cDNA); second, a duplex of synthetic oligonucleotides with sequences 5′-AGCTTGCGGCCGCCATGGTGAAATCCTCCCTGCAGCG and 5′-GATCCGCTGCAGGGAGGATTTCGACCATGGCGGCCGCA, compatible with HindIII and BamHI sites; and third, the larger EcoRI–HindIII fragment of pGEM4Z (Promega). The in-frame mutant lacking T205 (ΔT205) was produced by PCR-based site-directed mutagenesis (Higuchi et al., 1988). A plasmid with the BMV coat protein–antizyme fusion, C3NE, was prepared as follows. The HindIII–BamHI fragment of NE was first replaced by a duplex of synthetic oligonucleotides 5′-AGCTAGATCTGAGAAGCTTGGTGAAATCCTCCCTGCAGCG and 5′-GATCCGCTGCAGGGAGGATTTCACCAAGCTTCTCAGATCT. A fragment corresponding to nucleotides 1242–1816 of BMV RNA3 (Ahlquist et al., 1981) was amplified by PCR on the plasmid pB3TP7 (Janda et al., 1987) (provided by Dr. J. Bujarski) with two immediately flanking restriction sites, 5′-BglII and 3′-HindIII, introduced to the primers. The PCR fragment was digested with BglII and HindIII and inserted into the BglII and HindIII sites of the modified NE plasmid. 5′ deletion constructs were prepared by replacing the smaller HindIII–NheI fragment (279 nt) of C3NE with HindIII–NheI-digested PCR fragments on ZZ5 by use of 5′ primers with the sequence 5′-GGAAGCTTG, followed immediately by antizyme sequences, nucleotides 103–122, 154–174, or 196–214, and a 3′ primer complementary to nucleotides 317–337. To introduce various mutations at the shift site or the pseudoknot region, nucleotides 191 and 257 of C3NE (numbered as in Figure 1) were changed by PCR mutagenesis to C and T, respectively, creating ApaI and BclI sites. ApaI–BclI or ApaI–NheI fragments of this plasmid were then substituted by pairs of oligonucleotides with mutant sequences.

Details of construction of pGG75 will be described elsewhere and are available upon request (N. M. Wills, M. G. Sandbaken, J. F. A., and R. F. G., unpublished data). In brief, a PCR fragment containing 5′ to 3′, a BglII site, T7 promoter, the GST coding region (including the downstream thrombin recognition site from pGEX-2T [Pharmacia]), and a BstEII–XhoI–KpnI–BamHI polylinker was digested with BglII and BamHI and inserted into the BamHI site of the yeast expression vector pG-1 (Schena et al., 1991). In this construct, pGG75, GST is transcribed from either the GPD or T7 promoter.

pGB01 (Figure 6A) was constructed by introducing the rabbit β-globin gene into the unique BamHI site of pGG75. The fragment was prepared by PCR on pOGβ17 (Görlach et al., 1989, provided by Dr. J. Schickinger via Dr. M. F. Tuite) and contained the sequence from the second codon of β-globin through the BglII site 3 nt downstream to the end of the ORF, with a BamHI site introduced immediately 5′ of the second codon. Sequences of the antizyme frameshift window, corresponding to nucleotides 190–268, were amplified with PCR and inserted into the BstEII and KpnI sites of pGB01 (Figure 6A). The antizyme sequence was flanked by two As at 5′ and either GG (pGB-103, for monitoring +1/−2 frameshifting), CGG (pGB-T01, for −1 frameshifting) or G (pGB-R01, for readthrough) at 3′ between the restriction sites to adjust the reading frame. For monitoring −1 frameshifting, the nucleotide corresponding to A227 (Figure 1) was also replaced by a G. This changed the in-frame UGA codon to UGG and did not affect the pseudoknot formation. The construct used for amino acid sequencing in Figure 3, pGB-F01, is similar to pGB-103 and has an insert consisting of antizyme sequence 193–268 flanked by two oligonucleotides specifying factor Xa sites, 5′-AAATCGAAGGT at 5′ and 5′-ATTGAAGGTAGAATCGG at 3′.

For each construct used in this paper, two independent clones were verified by DNA sequencing, and both were subjected to expression experiments.

In Vitro Transcription and Translation

DNA templates for in vitro transcription were either linearized plasmids (digested with EcoRI) or PCR fragments of the plasmids amplified with an upstream primer, 5′-GGCTCGAGTAATACGACTCACTATAGGG, containing the T7 promoter sequence, and downstream primers complementary to the sequence corresponding to the end of the ORFs, which are followed by the tetranucleotide 5′-TAGG. Templates with these two methods gave identical results. RNAs were synthesized with T7 polymerase (Stratagene or Promega) according to the instructions of the supplier. Translation mixture (15 μl) contained 0.6 pmol of the mRNA, 15 μCi of [^{35}S]methionine (>1000 Ci/mmol), and 10.5 μl of ribonuclease-treated rabbit reticulocyte lysate (from Wako Chemicals for experiments in Figures 2A–2C or Promega for other experiments) with or without addition of polyamines. After incubation at 30°C for 45 min, 1 μl of 1 μg/μl RNase A was added, incubated for a further 10 min, and analyzed by SDS–PAGE. For immunoprecipitation (Kameji and Pegg, 1987), either 20 μg of anti-antizyme IgG (Matsufuji et al., 1990b) or control rabbit IgG or 2 μl of anti-BMV rabbit serum (ATCC) was added and incubated for 15 min. Protein A–Sepharose (Pharmacia, 40 μl of 50% slurry) was then added and mixed for 1 hr. The pellet was washed with 0.5 ml of immunoprecipitation buffer five times, suspended in sample buffer, heated, and subjected to SDS–PAGE.

Translation of albumin was carried out with 0.5 μg of poly(A)$^+$ RNA from rat liver followed by immunoprecipitation with anti-rat albumin (Kameji and Pegg, 1987). Gels were processed for fluorography by using Amplify (Amersham) in the experiments shown in Figures 2A–2C, or for autoradiography in the other experiments. The exposure times were 6–12 hr. The products were quantitated either by direct counting of excised bands or by scanning the gel on a PhosphorImager (Molecular Dynamics).

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